Growth of *Aeromonas hydrophila* K144 as Affected by Organic Acids

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ABSTRACT

The influence of different acids on the aerobic growth kinetics of *Aeromonas hydrophila* was studied in BHI broth with 0.5 and 2.0% NaCl incubated at 5 and 19°C. Growth curve data were analyzed by the Gompertz equation and a nonlinear regression program; generation and lag times were calculated from the Gompertz parameters. Type of acid, pH, NaCl level and temperature influenced lag and generation times. The organic acids (acetic, lactic, citric and tartaric) inhibited growth at higher pH values than inorganic acids (HCl and H_2SO_4). The high NaCl level interacted with type of acid and pH to restrict growth of the organism at the lower temperature of incubation. Acetic and lactic acids were effective in controlling the growth of *A. hydrophila* and could readily be combined with low holding temperature to render foods free of the organism.

Key Words: Microbes, Aeromonas, lactic acid, acetic acid, citric acid, tartaric acid

INTRODUCTION

AEROMONAS HYDROPHILA is a facultative, Gram-negative bacterium widely distributed in the environment, especially in water supplies (Hazen el al., 1978). It is also found in foods, including vegetables (Callister and Agger, 1986) and foods of animal origin (red meats, poultry, fish, and seafood) (Palumbo et al., 1985a; Okrend et al., 1987). The organism can readily grow in wide variety of foods held refrigerated at 5°C (Palumbo et al., 1985a; Callister and Agger, 1986). The role of *A. hydrophila* as a putative pathogen in humans has been extensively reviewed (Buchanan and Palumbo, 1985; Palumbo et al., 1991a).

Since *A. hydrophila* can readily grow in adequately refrigerated foods, inhibition of the organism must rely on a combination of factors such NaCl, low pH, nitrite and low temperature. Previous work by Palumbo et al. (1985b, 1991b) has developed combinations of pH, NaCl, and nitrite which prevented the growth of *A. hydrophila* at 5°C. In those studies, HCl was employed as the acidulant. However, foods contain other acids whether added directly during processing or via fermentation. The objective of our study was to investigate the effect of different acids (organic and inorganic) on the kinetics of aerobic growth of *A. hydrophila* K144 as a representative strain in culture broth containing 0.5 and 2% NaCl and incubated at 5 and 19°C.

MATERIALS & METHODS

Organism

Aeromonas hydrophila K144 was used as a representative strain. All experiment flasks were inoculated from a starter flask of brain heart infusion broth (BHI, Difco, Detroit, MI) grown overnight (18–20 hr) at 28°C and 150 rpm.

Variables

Sodium chloride was added to BHI to give a final concentration of 0.5 or 2.0% NaCl (W/V) with adjustment of the starting pH to 6.5, 6.0, 5.5, and 5.0 using either organic or inorganic acids as acidulants. The acids used were reagent grade HCl, H_2SO_4, acetic, lactic, citric, and tartaric. The first four acids were used to adjust the pH as received from the manufacturer; citric and tartaric were used as 10% solutions. With broth containing additional NaCl, pH was adjusted after addition of the salt. The experimental flasks contained 50 mL of culture broth/250 mL flask. A complete factorial experimental design was used: 2 temperatures × 2 NaCl levels × 6 acids × 4 pH levels.

Experimental procedure

Triplicate flasks of BHI (at either NaCl level and adjusted to different pH values with the various acids) were incubated at either 5 or 19°C after inoculation from the starter flask to yield a count at zero time of *ca* 10^3CFU/mL. Incubation was aerobic (150 rpm) in a New Brunswick Psychrotherm incubator (model G26). Counts were made at zero time as well as at intervals appropriate to the experimental culture conditions. During incubation, portions of culture were removed, diluted if necessary in 0.1% peptone water and surface plated (Spiral plater, Model D) onto tryptic soy agar (Difco Labs, Inc., Detroit, MI) and the plates were counted after 24 to 36 hr at 28°C using a Laser counting system (Spiral Systems, Bethesda, MD). Counts were transformed to $\log_{10}$ and growth data were then analyzed by the Gompertz equation (Gibson et al., 1987) using Abacus (an iterative, non-linear regression program). From the Gompertz parameters, values for lag and generation times were calculated for each variable combination.

RESULTS & DISCUSSION

WHILE analysis of growth data by the Gompertz equation can yield various kinetic parameters, we chose to present the influence of cultures conditions (acid type, pH, NaCl level, and temperature) in the form of generation times (Tables 1 and 3) and lag times (Tables 2 and 4). The data in Tables 1 and 4 are the result of analyzing 192 individual growth curves. In addition to generation and lag times, we also presented conditions (temperature, pH and acid type, and NaCl) under which *A. hydrophila* did not grow (NG): either the organism died off (viable count decreased from the starting count of *ca* 1×10^3 CFU/ml to undetectable (<21/ml)) (see Fig. 1) or the viable count remained constant. The number of viable *A. hydrophila* declined according to the type of acid; almost 300 hr were required to reach the undetectable level with HCl (Fig. 1), while with lactic and acetic only 24 to 48 hr were required to reach this point. The other acids were intermediate. In a few instances, the viable count remained stable for extended periods of incubation. (Both decline and stable viable counts are represented by NG in the Tables).

The results of our study (Tables 1 to 4) can be viewed in terms of how the variable combinations affected the lack of growth (no growth conditions), lag times, and generation times (GT). For NG conditions, pH 5.0 at 5°C (Table 1) was lethal at both low (Fig. 1) and high NaCl levels. At 5°C, acetic acid prevented growth at pH 6.0 and lactic acid at pH 5.5. This was undoubtedly related to their higher pK_a (see below). The extreme sensitivity of *A. hydrophila* to acetic acid may be related to the "suicide phenomenon" in which certain strains of mesophilic aeromonads are killed at the end of the normal growth cycle by self-produced acetic acid (Namdari and Cabelli, 1989). At 19°C, the organic acids were equally effective at preventing growth at pH 5.0 (Table 3); in contrast, the inorganic acids allowed growth (Table 3). The influence of the variable combinations on lag times (Tables 2 and 4) was not

Table 1—Effect of starting pH and NaCl level on the generation time of A. hydrophila *grown in different acids at 5°C*

	0.5% NaCl				2% NaCl			
	pH				pH			
acid	6.5	6.0	5.5	5.0	6.5	6.0	5.5	5.0
HCl	1.36 ± 0.10[a]	2.09 ± 0.12	7.83 ± 1.51	NG[b]	1.96 ± 0.81	2.65 ± 0.46	NG	NG
H_2SO_4	3.71 ± 0.58	4.38 ± 0.29	5.53 ± 0.24	NG	2.19 ± 0.08	2.06 ± 0.05	7.79 ± 1.90	NG
acetic	1.39 ± 0.05	NG	NG	NG	3.14 ± 0.98	8.44 ± 0.72	NG	NG
lactic	3.39 ± 0.21	4.28 ± 0.11	NG	NG	4.44 ± 0.20	3.96 ± 0.41	NG	NG
citric	1.85 ± 0.06	2.00 ± 0.17	3.59 ± 0.56	NG	3.26 ± 0.17	3.29 ± 0.16	NG	NG
tartaric	4.50 ± 0.30	5.14 ± 0.04	9.59 ± 0.37	NG	3.13 ± 0.12	3.24 ± 0.08	NG	NG

[a] Hr, average of three replicates, ± standard deviation.
[b] No growth.

Table 2—Effect of starting pH and NaCl level on the lag time of A. hydrophila *grown in different acids at 5°C*

	0.5% NaCl				2% NaCl			
	pH				pH			
acid	6.5	6.0	5.5	5.0	6.5	6.0	5.5	5.0
HCl	67.50 ± 0.53[a]	64.50 ± 0.33	74.64 ± 12.90	NG[b]	55.8 ± 15.90	83.6 ± 7.60	NG	NG
H_2SO_4	50.99 ± 5.61	45.21 ± 1.61	63.54 ± 0.57	NG	71.92 ± 3.70	87.72 ± 3.30	194.23 ± 18.40	NG
acetic	132.58 ± 0.54	NG	NG	NG	113.69 ± 15.0	439.33 ± 10.20	NG	NG
lactic	63.90 ± 3.90	102.17 ± 1.70	NG	NG	81.30 ± 5.60	199.22 ± 6.90	NG	NG
citric	44.35 ± 2.34	44.49 ± 2.15	58.68 ± 5.20	NG	49.28 ± 3.40	114.03 ± 2.70	NG	NG
tartaric	41.56 ± 2.49	30.71 ± 3.20	53.51 ± 3.23	NG	61.15 ± 2.80	73.45 ± 2.34	NG	NG

[a] Hr, average of three replicates, ± standard deviation.
[b] No growth.

Table 3—Effect of starting pH and NaCl level on the generation time of A. hydrophila *grown in different acids at 19°C*

	0.5% NaCl				2% NaCl			
	pH				pH			
acid	6.5	6.0	5.5	5.0	6.5	6.0	5.5	5.0
HCl	0.28 ± 0.01[a]	0.41 ± 0.05	0.67 ± 0.02	5.15 ± 0.53	0.62 ± 0.05	0.79 ± 0.06	1.14 ± 0.26	1.47 ± 0.06
H_2SO_4	0.69 ± 0.02	0.75 ± 0.01	0.78 ± 0.02	1.39 ± 0.10	0.79 ± 0.09	0.84 ± 0.23	1.02 ± 0.07	1.08 ± 0.03
acetic	1.64 ± 0.83	1.16 ± 0.08	NG[b]	NG	0.59 ± 0.07	2.03 ± 0.24	NG	NG
lactic	0.67 ± 0.05	1.00 ± 0.38	NG	NG	0.65 ± 0.30	0.96 ± 0.09	NG	NG
citric	0.76 ± 0.04	0.70 ± 0.04	0.77 ± 0.04	NG	0.45 ± 0.09	0.77 ± 0.04	1.34 ± 0.18	NG
tartaric	0.69 ± 0.04	0.67 ± 0.03	0.66 ± 0.05	NG	1.11 ± 0.03	1.52 ± 0.15	1.09 ± 0.28	NG

[a] Hr, average of three replicates, ± standard deviation.
[b] No growth.

Table 4—Effect of starting pH and NaCl level on the lag time of A. hydrophila *grown in different acids at 19°C*

	0.5% NaCl				2% NaCl			
	pH				pH			
acid	6.5	6.0	5.5	5.0	6.5	6.0	5.5	5.0
HCl	16.59 ± 2.08[a]	18.64 ± 0.42	21.79 ± 0.05	92.31 ± 5.10	8.29 ± 0.27	6.95 ± 0.24	8.38 ± 1.21	37.40 ± 1.88
H_2SO_4	7.12 ± 0.17	7.43 ± 0.25	9.13 ± 0.15	16.40 ± 2.30	7.43 ± 0.88	13.29 ± 2.15	12.29 ± 0.61	42.27 ± 0.28
acetic	12.23 ± 2.14	90.24 ± 2.58	NG[b]	NG	17.44 ± 0.63	42.09 ± 0.71	NG	NG
lactic	10.53 ± 0.98	10.73 ± 0.68	NG	NG	25.90 ± 1.30	23.80 ± 1.08	NG	NG
citric	7.97 ± 0.09	7.95 ± 0.60	24.59 ± 0.98	NG	16.64 ± 0.29	18.15 ± 1.35	26.60 ± 2.73	NG
tartaric	8.11 ± 0.50	7.88 ± 0.29	20.49 ± 0.49	NG	7.72 ± 2.14	7.75 ± 1.49	26.01 ± 3.58	NG

[a] Hr, average of three replicates, ± standard deviation.
[b] No growth.

as clearly dependent on acid type (organic *vs* inorganic) as was the effect on no growth conditions. However, lag times increased as pH decreased for each acid and lag times were longer for cultures with 2% NaCl at both temperatures. As expected, lag times increased at 5° compared to 19°C.

The influence of the variable combinations on GT (Tables 1 and 3) very much duplicated the effect on lag times. GT increased with decreasing temperature, decreasing pH, and increased NaCl level. The variables did not appear to have any influence on the maximum population density (MPD) (data now shown). The MPD for all experiments shown in the Tables was $\log_{10} = 10.87 \pm 0.53$.

The data (Table 1 to 4) represent the first complete analysis of the effect of acid type on aerobic growth of *A. hydrophila*. In general, low pH (5.0) was more inhibitory when combined with low temperature (5°C) and higher salt level (2%). Further, as expected from studies of organisms such as *Yersinia enterocolitica* (Brocklehurst and Lund, 1990), organic acids were more inhibitory than inorganic acids. Freeze et al. (1973) indicated that the inhibition of bacteria by weak acids (such as those we used) was related to the concentration of undissociated acid. The pK_a (pH at which 50% dissociation occurs) for the acids studied were: acetic (4.76), lactic (3.86), citric (3.4 [K_1] and 4.77 [K_2]), and tartaric (2.98 [K_1] and 4.34 [K_2]) (Dziezak, 1990). Acetic acid, with the highest pK_a of the organic acids tested, was most inhibitory. With the other organic acids tested, the relationship between pH_a and inhibition was not as distinct and other bases for inhibition may apply.

In summary, our results provide information on the influence of different acidulants on the kinetics of aerobic growth of *A. hydrophila* and on combinations which completely prevented growth of the organism. They also extend previous work on the effect of pH, salt, and temperature on the growth of the organism (Palumbo et al., 1985b, 1991b). In general, low pH was more effective in inhibiting the growth of *A. hydrophila* when combined with low temperature and added NaCl.

Differences were found among the acids tested, with acetic

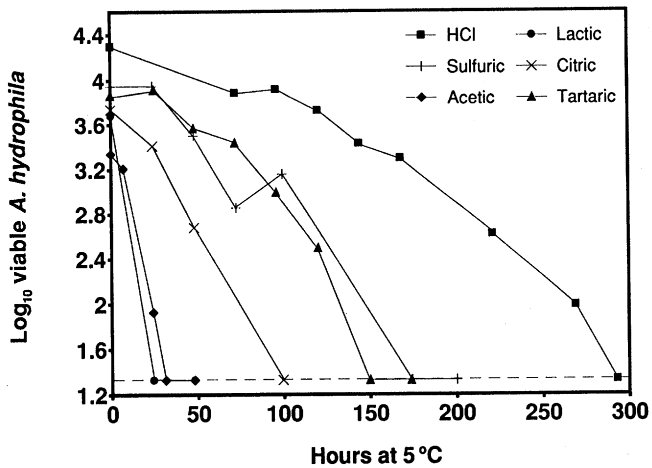

Fig. 1—Effect of different acids [HCl, sulfuric, tartaric, citric, acetic, and lactic] on the decline in viable count of A. hydrophila *in BHI broth at pH 5.0 and 5°C (0.5% NaCl). [dashed line-lower limit of detection (log_{10} = 1.33)]*

acid being the most effective, particularly at inhibiting growth at a relatively high pH, pH 6.0. Our results suggested that the growth of the organism in various foods could readily be controlled by an acetic acid treatment. The conditions for the inhibitory effect of lactic acid suggested that *A. hydrophila* should not be a problem in fermented foods such as Lebanon bologna and summer sausage, yogurt and various cheeses, and sauerkraut.

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